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PRINCIPAL INVESTIGATOR: Jamie N. Holloway

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20007

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6. AUTHOR(S)
Jamie N. Holloway

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20007

E-Mail: hollowaj@georgetown.edu

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13. ABSTRACT (Maximum 200 Words)
Estrogen receptor a (ERα) negative breast tumors often overexpress growth factor receptors, resulting in increased growth factor signaling and hyperactivation of MAPK (ERK1 and ERK2). We have previously shown that ERα-positive MCF-7 cells engineered to stably overexpress various signaling molecules leading to MAPK hyperactivation lose expression of ERα without inducing its transcriptional activation. The downregulation of ERα in these cells is transcriptional and is a specific action of MAPK hyperactivation that is reversible by MAPK abrogation. Here, we show that downregulation of ERα is not mediated specifically by either ERK-1 or -2. TAM67, a construct preventing AP-1 transcriptional activity, was used to determine that AP-1 activity does not play a role in ER downregulation. AP-1 activity is upregulated in response to MAPK activation, and increased AP-1 activity has been observed in ERα negative and hormone independent breast cancers. However, these are the first data indicating mechanistically that despite data correlating increased AP-1 activity with hormone independence/ERα-negativity, increased AP-1 activity is not responsible for ERα downregulation. Use of a dominant negative RSK1 construct indicates that RSK1 activity does not downregulate ERα. Transfection of ERK2Δ19-25, which is dominant negative for nuclear MAPK substrates while allowing activation of cytoplasmic substrates, revealed that a cytoplasmic substrate of MAPK is responsible for the generation of the ERα-negative phenotype in these cells. Collectively, these data reveal that the association between increased AP-1 activity and the ERα-negative phenotype is correlative, not causative, and that a cytoplasmic MAPK substrate other than RSK1 is responsible for ERα downregulation in our cell line models.

14. SUBJECT TERMS
Signal transduction, MAPK, AP-1, ERα, tamoxifen resistance

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Introduction

Upon diagnosis, breast cancer is described as either estrogen receptor (ER)-positive or ER-negative. Patients with ER-positive tumors have a longer disease free and overall survival, and they respond better to hormonal therapies such as tamoxifen, which is easier to tolerate than cytotoxic chemotherapy [1]. Conversely, patients with ER-negative tumors tend to have more aggressive disease and must be relegated to much harsher chemotherapy regimens [2,3]. Unlike ER-positive tumors, ER-negative tumors tend to overexpress growth factor receptors such as EGFR and c-erbB-2, and they have been shown to have high levels of activation of downstream signaling molecules such as MAPK [4-6]. Previous studies indicated that the hyperactivation of MAPK is directly responsible for the downregulation of ER in breast cancer cells, and that this downregulation is reversible via abrogation of MAPK activity [7]. Consequently, the present study seeks to identify the mechanism of this MAPK induced phenomenon. The outcome of this study has the potential to impact the lives of breast cancer patients who may be able to benefit from a treatment protocol where the blocking of growth factor signaling through MAPK can return ER expression and tamoxifen sensitivity, allowing ER-negative patients to avoid the harsh side effects of cytotoxic chemotherapy.

Body

Statement of Work

Task 1. Identify whether MAPK-induced downregulation of ERα is mediated specifically by ERK1 or ERK2. (months 1-8)

- *Overexpress ERK1 or ERK2 using activated, wild type ERK constructs*

Wild type ERK constructs were obtained from Melanie Cobb. While data from Dr. Cobb's lab indicated that these wild type constructs were fully activated by serum (personal communication), when these constructs were overexpressed in ER-positive MCF7 breast cancer cells in the presence of serum, they did not appear to be active. Phospho-MAPK western blots, as well as western blots for downstream effectors activated by active MAPK, showed no increase in activity with the expression of these constructs. Therefore, abrogation with dominant negative constructs would be the most informative experiment to determine impact of ERK signaling.

- *Abrogate ERK1 and ERK2 mediated signaling via dominant negative ERK1 and ERK2 constructs*

Overexpression of both dominant negative ERK 1 and dominant negative ERK2 together resulted in the return of ER at the highest level in all three ER-negative cell lines, while overexpression of either construct alone was also sufficient to return ER activity (Figure 1). Therefore, to the extent that can be presently assessed, the downregulation of ER by high MAPK activity does not appear to be mediated specifically by either ERK.

Task 2. Identify the role of AP-1 and its composition in ERα downregulation. (months 6-18)

- *Determine AP-1 composition in ERα-negative and ERα+ cell lines using fos and jun family member-specific antibodies by Western blotting and antibody supershifting*

Pending final results of the second part outlined in Task 2, this section has not been completed. As preliminary data indicate that abrogation of AP-1 activity does not play a role in the downregulation of ER in these model cell lines, this set of experiments may be omitted. However, Santa Cruz makes a series of antibodies against all fos and jun family members that can be easily obtained should the need arise.

- *Abrogate AP-1 expression using a dominant negative jun construct, Tam67*

The Tam67 construct was obtained from Powell Brown, and is used to abrogate all AP-1 driven transcription. Overexpression of this construct in the ER-negative model cell lines does not result in the reversal of ER downregulation (Figure 2). Therefore, while there is significant clinical data correlating high levels of AP-1 activity with ER-negativity and hormone independence [8-10], these are the first data to demonstrate that high levels of AP-1 activity do not directly result in the downregulation of ER in breast cancer.

Task 3. Assess the role of cytoplasmic substrates of MAPK in ERα repression. (months 18-36)

- *Determine the localization of the key MAPK substrate*

The ERK2Δ19-25 construct [11] was obtained from Dr. Michael Weber. Because it lacks the domain for association with MEK, it localizes preferentially to the nucleus without becoming activated. This prevents the activation of nuclear MAPK substrates while allowing the activation of substrates by endogenous MAPK in the cytoplasm. (It acts as a dominant negative for nuclear substrates only.) Transient overexpression of this construct in the ER-negative model cell lines did not result in a reversal of ER repression (Figure 3). These data indicate that a cytoplasmic substrate of MAPK is responsible for the downregulation of ER in these model cell lines.

- *Compare $pp90^{RSK}$ activity levels in ERα-negative and ERα+ cell lines using ant anti-phospho-$pp90^{RSK}$ antibody*

A Cell Signaling antibody raised against the activiating phosphorylation of pp90RSK was used to identify the level of RSK activity in the ER-positive and -negative model cell lines (Figure 4a). Analysis of whole cell lysates revealed that the level of pp90RSK activity correlates with the level of MAPK activity.

- *Generation of $pp90^{RSK}$ constructs*

A constitutively active RSK construct has been obtained from Dr. Jeffrey Smith, and a dominant negative RSK construct has been obtained from Dr. John Blenis, so the construction of any constructs will not be necessary for the completion of this task.

- *Determine if $pp90^{RSK}$ overexpression causes ERα downregulation in ERα+ cell lines*

The dominant negative RSK construct, obtained from Dr. John Blenis, was overexpressed in the ER-negative model cell lines. When transiently overexpressed in the ER-negative cell line models, it did not result in the return of ER activity (Figure 4b), therefore, pp90RSK is not responsible for the downregulation of ER in this system.

- *Determine if AIB1 (activated in breast cancer-1) plays a role in ERα downregulation in ERα+ cell lines*

As the ERK2Δ19-25 construct indicated that a cytoplasmic substrate was responsible for ER downregulation, but data shown in Figure 5 indicate that this substrate is not RSK, further cytoplasmic substrates were sought. AIB1 is a steroid receptor coactivator in the p160 SRC family that coactivates transcription of ER regulated genes. It has recently been identified to be a substrate of MAPK [12], and has been shown to localize to the cytoplasm in its unphosphorylated state [13]. Therefore, AIB1 is a cytoplasmic substrate of MAPK. Our lab studies AIB1, and as such, many reagents for the study of its interaction with ER expression are available. Specifically, I will employ the use of siRNA against AIB1 to determine the effect of AIB1 depletion on ER-positive cells. In addition, we have an AIB1 antibody (Transduction Labs), which will allow me to assess whether any correlation exists between AIB1, MAPK, and ER expression in our model cell lines. We are also in possession of various tagged AIB1 constructs which I could use for further study should preliminary experiments warrant. Characterization of the interaction between AIB1 and ER expression will serve to complete Task 3.

Key Research Accomplishments

- Determination that the abrogation of either ERK1 or ERK2 or both ERKs in combination leads to the reversal of ER downregulation
- Determination that abrogation of AP-1 mediated transcription does not reverse ER downregulation in ER-negative model cell lines
- Determination that a cytoplasmic substrate of MAPK other than pp90RSK is responsible for the downregulation of ER
- Obtained key reagents (including AIB1 siRNA, antibody, and expression vectors) for the completion of Task 3

Reportable Outcomes

Abstracts

Murthy, S., **Holloway, J.N.,** and El-Ashry D. Identification of MAP kinase substrates responsible for the downregulation of ERα in breast cancer cells. 94th Annual Meeting of the American Association for Cancer Research, Washington, DC, 2003, Abstract #1953

Holloway, J.N., Alexander, J., and El-Ashry, D. A Substrate of MAPK is Responsible for the Downregulation of ERα in Breast Cancer Cells. 93rd Annual Meeting of the American Association for Cancer Research, San Francisco, CA, 2002. Abstract # 5332.

Conclusions

Previous data indicated that hyperactivation of MAPK results in the downregulation of ER in ER-positive breast cancer cells, and that this downregulation is reversible through the abrogation of both ERK1 and ERK2, either through MEK inhibition with U0126, or through the use of dominant negative constructs. We have now demonstrated that this ER downregulation is not a result of a specific substrate of either ERK1 or ERK2, as abrogation of either ERK or a combination of the two will result in the return of ER in ER-negative cells. As AP-1 family members are key MAPK substrates, we examined the effect of AP-1 abrogation on ER-negative cell lines to determine if clinical data correlating high AP-1 activity with ER-negativity had a causative relationship. Our data indicate that high AP-1 activity does not result in the downregulation of ER in our model cell lines, and this is the first data demonstrating that while there is significant clinical data correlating ER-negativity with high AP-1 activity, this AP-1 activity is not responsible for the downregulation of ER and acquisition of hormone independence. Experiments with the ERK2Δ19-25 construct revealed that the substrate of MAPK responsible for the downregulation of ER resides in the cytoplasm. In addition, use of the dominant negative RSK construct provided data indicating that RSK is not the responsible cytoplasmic substrate. Future experiments for the completion of this project will entail the identification of any relationship between the phosphorylation of AIB1 by MAPK and the expression of ER. The use of siRNA, various tagged expression vectors, and an AIB1 antibody will facilitate the completion of the final task. Determining the identity of the MAPK substrate that is responsible for ER downregulation may enable

ER-negative patients to be treated with an inhibitor of that specific molecule, returning ER expression and tamoxifen sensitivity, allowing them to be treated with hormonal therapy and forgo the side effects that accompany cytotoxic chemotherapy.

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Appendices

Figures 1 through 5 follow.

Figure 1.

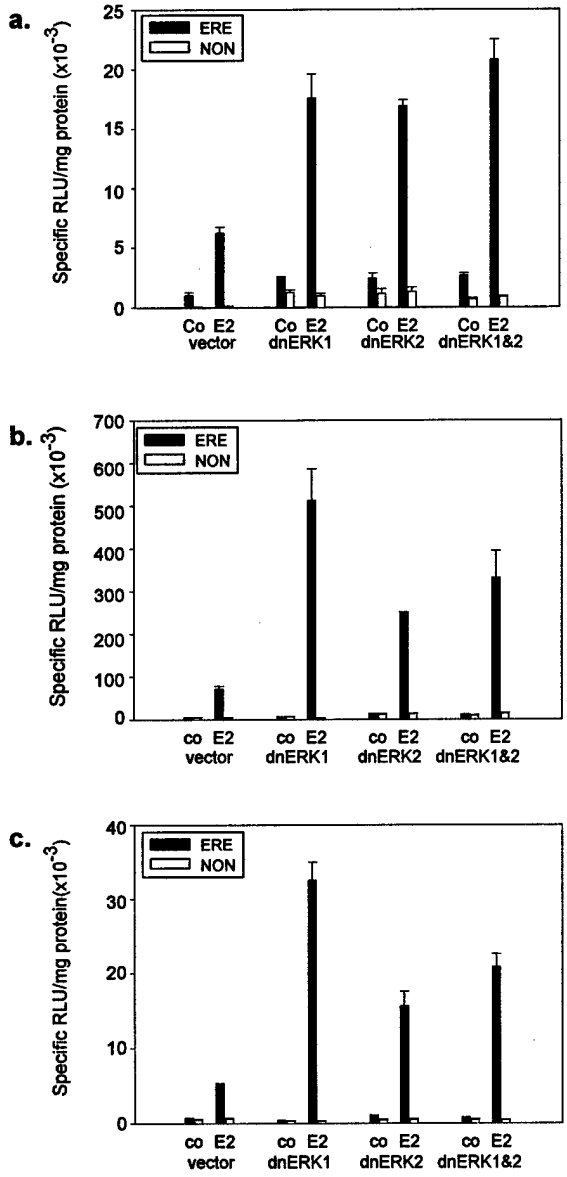

Figure 1. Downregulation of ER is not mediated exclusively by either ERK1 or ERK2. A.) Raf14c, B.) Mek15c, and C.) MB3 cells were transiently co-transfected with 1.25μg total dnERK constructs and 0.75μg luciferase reporter constructs and were treated with control (co) or estrogen containing (E2) media. ERE-luciferase is measured representing ER activity, the NON-luciferase construct is an identical plasmid, except that the ERE was scrambled to result in a nonsense sequence. Experiments are representative of at least three individual experiments, each done in triplicate. Error bars represent s.e.m.

Figure 2.

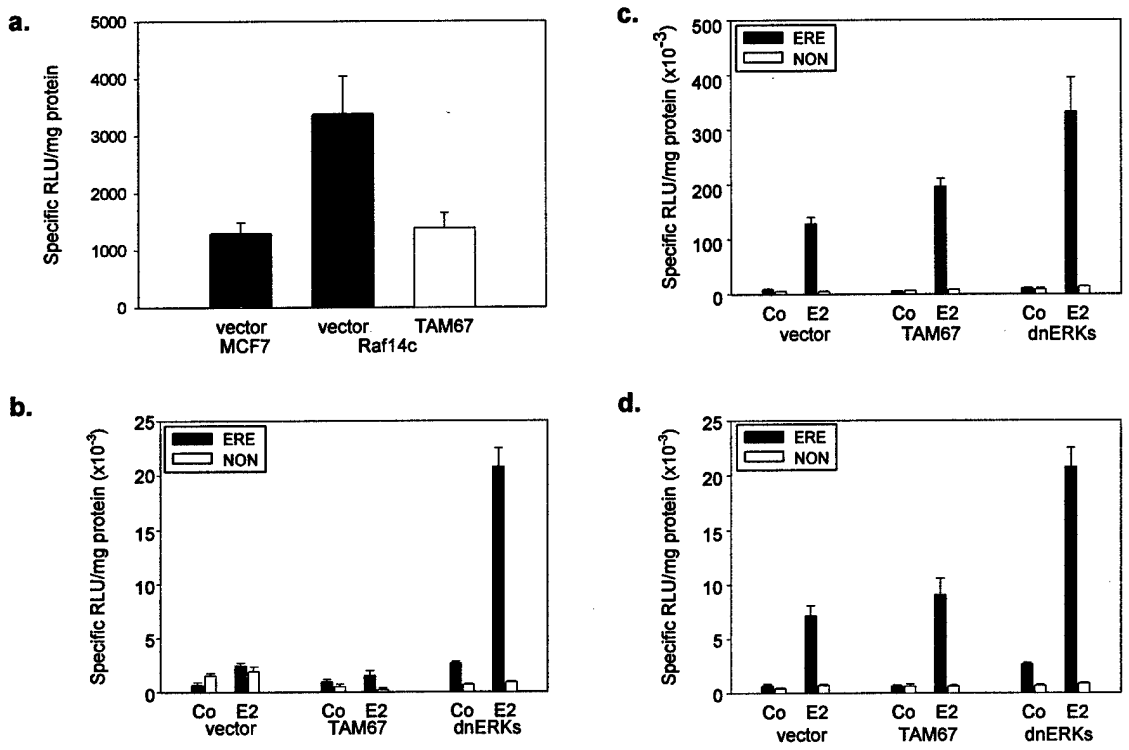

Figure 2. Downregulation of ER is not mediated by AP-1 activity. A.) *Expression of TAM67 inhibits AP-1 activity.* Raf14c cells were transiently transfected with 1.25μg TAM67 or vector control DNA and .75μg AP-1 luciferase reporter construct, and were treated with control media following transfection. This experiment is a representative figure, similar inhibition of AP-1 activity by TAM67 was found in multiple cell lines. B.) Raf14c, C.) Mek15c, and D.) MB3 cells were transiently co-transfected with 1.25μg TAM67, a dominant negative jun construct and 0.75μg luciferase reporter constructs and were treated with control (co) or estrogen containing (E2) media. ERE-luciferase is measured representing ER activity, the NON-luciferase construct is an identical plasmid, except that the ERE was scrambled to result in a nonsense sequence. Experiments are representative of at least three individual experiments, each done in triplicate. Error bars represent s.e.m.

Figure 3.

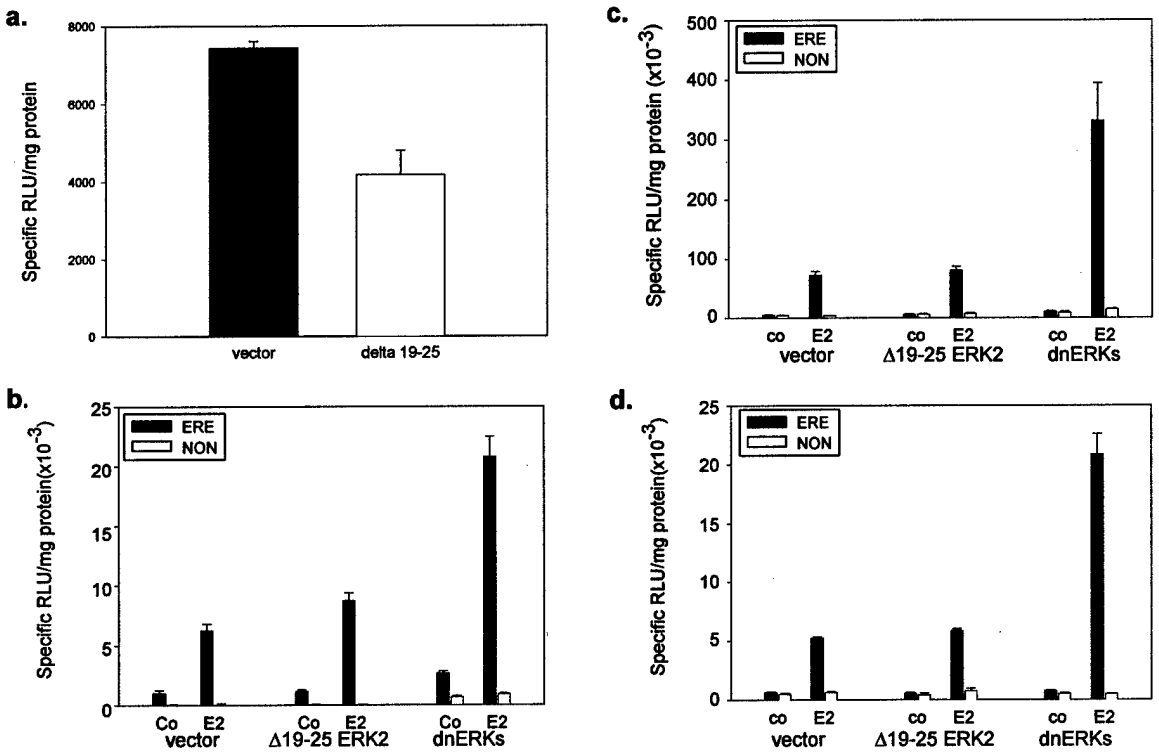

Figure 3. Downregulation of ER is mediated by a cytoplasmic substrate of MAPK. A.) *ERK2Δ19-25 represses activity of a nuclear MAPK substrate* Raf14c cells were transiently transfected with 0.625μg ERK2Δ19-25 or vector control DNA, 0.625μg pFAelk, and .75μg pFA-luciferase reporter construct, and were treated with control media following transfection. This experiment is a representative figure, similar inhibition of AP-1 activity by TAM67 was found in multiple cell lines. B.) Raf14c, C.) Mek15c, and D.) MB3 cells were transiently co-transfected with 1.25μg ERK2Δ19-25 construct and 0.75μg luciferase reporter constructs and were treated with control (co) or estrogen containing (E2) media. ERE-luciferase is measured representing ER activity, the NON-luciferase construct is an identical plasmid, except that the ERE was scrambled to result in a nonsense sequence. Experiments are representative of at least three individual experiments, each done in triplicate. Error bars represent s.e.m.

Figure 4.

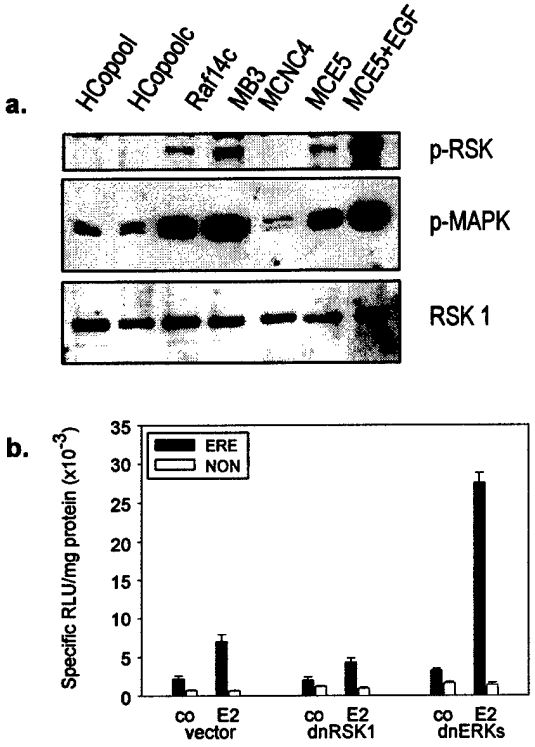

Figure 4. Downregulation of ER is not mediated by RSK1. A.) *RSK1 activity correlates with MAPK activity in breast cancer cells.* Whole cell lysates were prepared from cells in normal culture conditions grown to approximately 80% confluence. MCE5 cells were treated or not with 10 ng/mL for 10 minutes. Western blots were performed on 5μg of total protein for phospho-RSK1 and phospho-MAPK. A western blot for RSK1 is shown as a loading control. B.) Raf14c cells were transiently co-transfected with 1.25μg dnRSK1 construct and 0.75μg luciferase reporter constructs and were treated with control (co) or estrogen containing (E2) media. ERE-luciferase is measured representing ER activity, the NON-luciferase construct is an identical plasmid, except that the ERE was scrambled to result in a nonsense sequence. Experiments are representative of at least three individual experiments, each done in triplicate. Error bars represent s.e.m.